MOLECULAR AND CELLULAR BIOLOGY, Dec. 1983, p. 2271–2279 Vol. 3, No. 12
0270-7306/83/122271-09$02.00/0

Retinoic Acid-Induced Neural Differentiation of Embryonal Carcinoma Cells

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Received 29 March 1983/Accepted 13 September 1983

We have previously shown that the P19 line of embryonal carcinoma cells develops into neurons, astroglia, and fibroblasts after aggregation and exposure to retinoic acid. The neurons were initially identified by their morphology and by the presence of neurofilaments within their cytoplasm. We have more fully documented the neuronal nature of these cells by showing that their cell surfaces display tetanus toxin receptors, a neuronal cell marker, and that choline acetyltransferase and acetyl cholinesterase activities appear coordinately in neuron-containing cultures. Several days before the appearance of neurons, there is a marked decrease in the amount of an embryonal carcinoma surface antigen, and at the same time there is a substantial decrease in the volumes of individual cells. Various retinoids were able to induce the development of neurons in cultures of aggregated P19 cells, but it did not appear that polyamine metabolism was involved in the effect. We have isolated a mutant clone which does not differentiate in the presence of any of the drugs which are normally effective in inducing differentiation of P19 cells. This mutant and others may help to elucidate the chain of events triggered by retinoic acid and other differentiation-inducing drugs.

During embryogenesis, pluripotent cells become committed to specific differentiation pathways. Murine embryonal carcinoma (EC) cell lines provide a culture system with which to investigate this process of cellular determination (10) because EC cells can be maintained in the undifferentiated state in vitro by frequent subculturing (7, 15, 29), and they can be induced to differentiate into embryonic and extra-embryonic cell types by a variety of procedures, including aggregation and treatment with various drugs (12, 21, 22, 27, 34, 36, 37).

Our recent experiments involved the euploid P19 line of EC cells (24). These multipotent cells can contribute to many normal tissues in the developing embryo after transfer into mouse blastocysts (30). When P19 cells are aggregated in vitro in normal culture medium, most cells retain the EC form, and only a small proportion differentiate into extra-embryonic endoderm-like cells (14). If the aggregates are exposed to nontoxic concentrations of dimethyl sulfoxide (DMSO), differentiation is extensive, and large amounts of skeletal and cardiac muscle appear, although neurons and glia are absent (23). On the other hand, aggregates of P19 cells treated with retinoic acid (RA) at high concentrations (greater than 5×10^{-8} M) develop into neurons, glia, and fibroblasts, but not muscle (14). This paper contains a further analysis of the neural differentiation induced by RA treatment of P19 aggregates.

MATERIALS AND METHODS

Cell lines and culture techniques. The P19 line of EC cells was isolated from a teratocarcinoma induced in the C3H/He strain of mice. The cells are euploid with a normal male karyotype (24) and were cultured in alpha minimal essential medium (35) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2.5% fetal calf serum and 7.5% calf serum (Flow Laboratories, Mississauga, Ontario, Canada). They were maintained at 37°C in a 5% CO_2 atmosphere.

Differentiation was carried out as follows. Cells in the exponential growth phase were treated with Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.025% trypsin and 1 mM EDTA to remove them from the surface of the tissue culture dish. They were plated at a concentration of 10^5 cells per ml into a bacteriological-grade petri dish (22), where they aggregated spontaneously. The medium was replaced after 3 days, and 2 days later the aggregates were plated into tissue culture dishes. Aggregates were scored for the presence of neurons at 7 to 8 days. Immunofluorescence assays for tetanus toxin receptors and glial fibrillar protein (GFP) were carried out at 12 to 14 days.

RA (Sigma Chemical Co., St. Louis, Mo.) and the

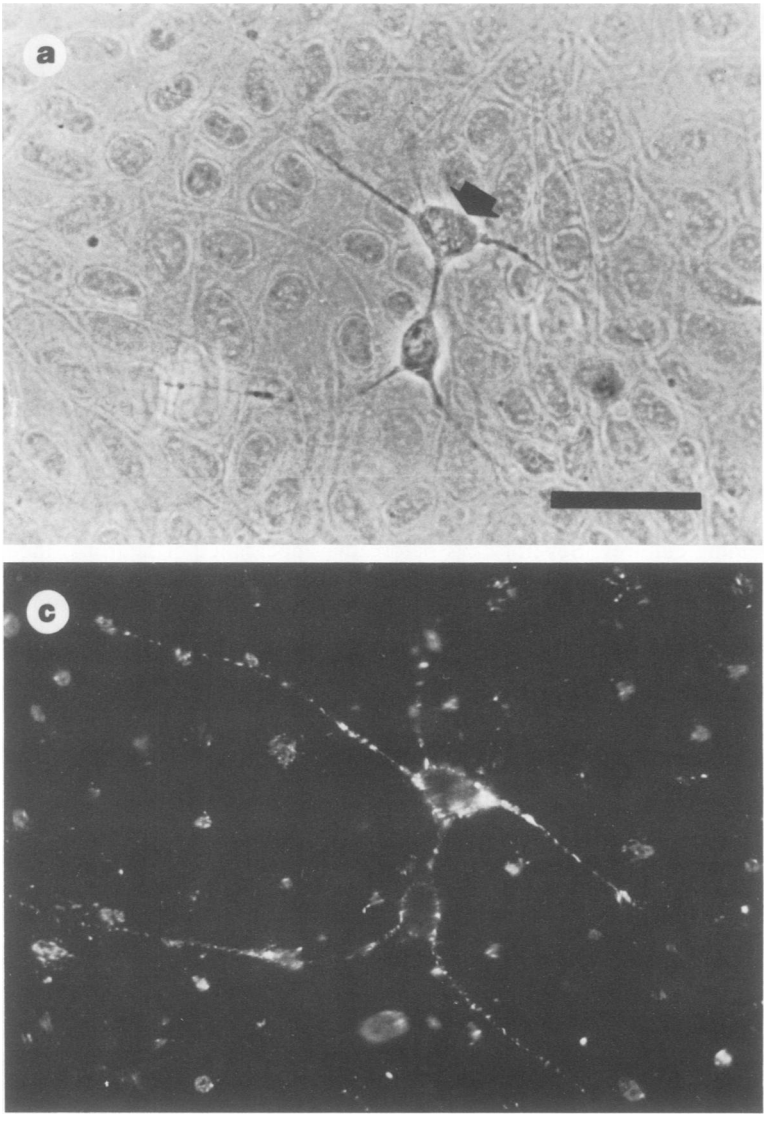

FIG. 1. Tetanus toxin and anti-GFP antiserum labeling of two different cell populations in RA-treated cultures of P19 cells. All panels show the same field of cells after double labeling with tetanus toxin and anti-GFP antiserum. The neurons are lying over a layer of flat glial cells. (a) and (c) were photographed at a higher focal plane than (b) and (d). (c) shows the cells photographed with rhodamine filters, which allow tetanus toxin binding to be visualized. The surface of the neuronal cell bodies and processes are clearly labeled in a patchy fashion characteristic of tetanus toxin labeling. (There is some randomly scattered fluorescence associated with the flat cells.) (a) is the corresponding phase micrograph. The staining of neurons was not observed if the tetanus toxin absorption step was omitted, and no staining of astroglia occurred on omission of the anti-GFP antiserum. Glial intermediate filaments are present in most of the flat cells, but the neurons are clearly not stained with the anti-GFP antiserum (d). The arrows in (a) and (b) indicate the position of one of the neurons. Bar, 50 μm.

other retinoids were prepared as 10^{-2} M stock solutions in ethanol and were diluted directly into the culture medium to obtain the desired concentration, usually 5×10^{-7} M.

Isolation of nonresponsive mutants. Mutant cells which did not differentiate in the presence of RA were isolated by a two-step procedure from the P19S18 cell line, a subclone of P19 (14). Initially, P19S18 cells were cultured in medium supplemented with 10^{-7} M RA. The cells were subcultured and maintained at subconfluent densities for 2 weeks. Undifferentiated cells were selected because of their relatively rapid growth rate. After plating at low density for a further 10 days, colonies of morphologically undifferentiated

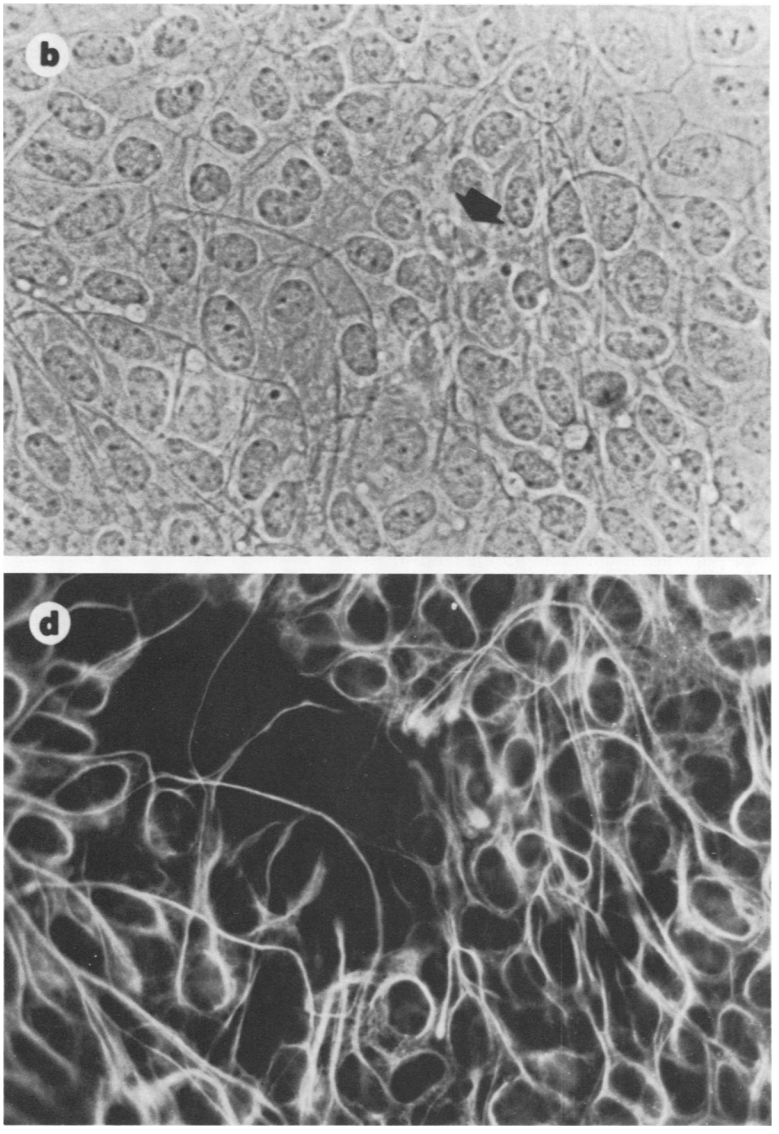

FIG. 1. *Continued.*

cells were obtained, one of which was expanded into a cell line and called P19S18RAC6. P19S18RAC6 was subjected to a second selection step in the presence of 10^{-5} M RA for 3 weeks. These cells were plated at low density for an additional week in the presence of the drug, and one of the colonies, P19S18RAC65 (RAC65), was grown up for further study.

Immunofluorescence labeling of neurons and glia. Aggregates of RA-treated and untreated cells were plated directly onto cover slips, and the cultures were allowed to develop undisturbed for 7 to 9 days. After washing in alpha medium, the cultures were incubated at room temperature for 30 min with 50 μl of tetanus toxin diluted 1:20 (Connaught Research Laboratories, Willowdale, Ontario, Canada) (25). After washing, the cover slips were treated with horse anti-tetanus toxin (Connaught) at a 1:50 dilution for 30 min, washed, and exposed to rhodamine-conjugated goat anti-horse immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.) at a 1:50 dilution for 30 min. The cover slips were then washed and fixed in 5% acetic acid in methanol for 15 min at −20°C. Subsequent staining with rabbit anti-GFP antiserum was as previously described (14).

The cover slips were mounted in 50% glycerol and were examined immediately with a Leitz photomicroscope equipped with epifluorescent optics.

EC antigen assays. For immunofluorescence experiments, aggregates were dissociated in 1 mM EDTA in PBS. The cells were allowed to settle for 10 min at unit

gravity onto cover slips previously coated with poly-L-lysine (1 mg/ml in water). After being washed in PBS, they were treated on ice with a 1:25 dilution of ascites fluid from hybridoma AEC3A1-9 (J. F. Harris, J. Chin, M. A. S. Jewett, M. Kennedy, and R. M. Gorczynski, submitted for publication) for 30 min. The cover slips were washed and treated with fluorescein-conjugated rabbit IgG raised against mouse IgM (Cedarlane Laboratories, Hornby, Ontario, Canada) diluted 1:5. The cells were fixed in 100% methanol at −20°C and then were stained with ethidium bromide (1 μg/ml in PBS) and scored immediately. Cells were scored as positive even if they had only one patch of fluorescence associated with their cell surface.

Quantitative absorption analysis was performed with gluteraldehyde-fixed cells by a modification of the procedure of Harris et al. (submitted for publication). The supernatants from samples of AEC3A1-9 antibody-treated cells were tested for residual activity in a two-step binding assay with fixed F9 EC cells and ^{125}I-labeled F(ab')$_2$ rabbit anti-mouse Fab. The cell concentration required to reduce the AEC3A1-9 activity by 50% (D_{50}) was derived from the cell titration data for each cell population and was normalized to the D_{50} for a control P19 culture. This method assumes a linear relationship between the relative D_{50} and the amount of antigen per cell.

Cells were prepared for gel electrophoresis and Western blot analysis by extraction in 0.5% Nonidet P-40. After electrophoresis in polyacrylamide gels, the resolved glycoproteins were transferred to nitrocellulose sheets, and the replica was incubated in AEC3A1-9 ascites fluid followed by exposure to ^{125}I-labeled F(ab')$_2$ rabbit anti-mouse Fab (Harris et al., submitted for publication). The lanes were identified by autoradioaugraphy, and pieces of the nitrocellulose replica were counted in a Beckman Gamma 4000 counter.

Estimation of median cell volume. We estimated the median cell volume from the size distribution obtained from a Coulter Counter Channelyser (Coulter Electronics Inc., Hialeah, Fla.) Our estimation of the median cell volume was the channel midway between the two channels containing 50% of the peak number of cells. Since the distributions were somewhat skewed, this estimated value was larger than the peak.

Enzyme assays. The cells were scraped with a rubber policeman from the tissue culture dishes, washed twice in PBS, and stored at −80°C. Before assay, all samples were suspended in an equal volume of water and were sonicated. Protein concentrations were determined by the Hartree modification of the Lowry procedure (11).

Choline acetyltransferase (CAT) was assayed by the radiochemical method described by Fonnum (9). Eserine, an esterase inhibitor, was added to each reaction mixture to prevent degradation of acetylcholine. Acetylcholinesterase (AchE) was added to a duplicate reaction mixture to determine the activity specifically attributable to the formation of acetylcholine.

The spectophotometric method of Ellman et al. (5) was used to assay AchE. The activity specifically due to AchE was determined by (i) adding a specific inhibitor of AchE, BW 284C51 (Sigma), and (ii) adding ethopropazine, which specifically inhibits pseudoesterases. The two values were averaged. Both inhibitors were kept in a stock solution at 10^{-2} M at 5°C and were used a final concentration of 10^{-5} M.

RESULTS

Characteristics of the neurons. The neurons which appeared in RA-treated cultures were identified in previous experiments by their form and their intermediate filaments, which could be specifically stained with antiserum directed against the 160,000-molecular-weight neurofilament protein (14). Another neuronal cell marker is the ability to bind tetanus toxin (1). Tetanus toxin binds to neurons in central nervous system explant cultures via specific cell surface gangliosides (2). Nonneuronal cells in the same cultures do not bind tetanus toxin (25).

The binding of tetanus toxin to neurons in RA-treated cultures was visualized by an indirect immunofluorescence assay. Figure 1a is a phase-contrast micrograph of a portion of an RA-treated aggregate. The neurons are lying on a monolayer of flat cells. Both the neuronal cell

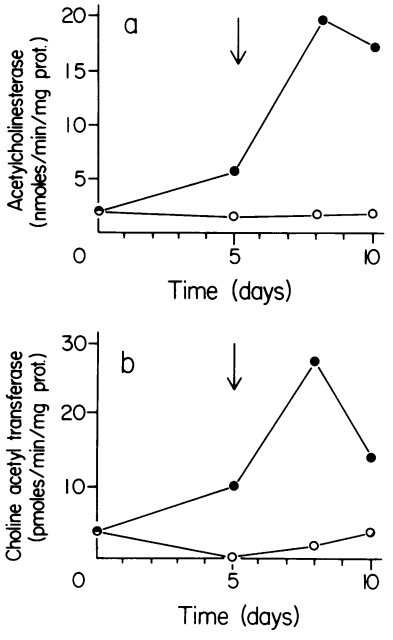

FIG. 2. Coordinate rise in CAT and AchE activities in RA-treated cultures. The specific activities of CAT and AchE were determined in treated (●) and untreated (○) aggregate cultures of P19 cells. Aggregates were plated at 5 days (arrow), and neurons became abundant at 7 days. (a) Each point represents an average of the specific activity of AchE which was specifically inhibited by BW 284C51 and the specific activity remaining after ethopropazine was added to the reaction mixture to inhibit pseudoesterases. Adult C3H/He mouse brain extracts contained a specific activity of 120 nmol per min per mg of protein. (b) Each point represents the specific activity of CAT obtained in the presence of eserine, an esterase inhibitor. An activity of 40 pmol per min per mg of protein was found in adult mouse brain.

bodies and their processes bound tetanus toxin (Fig. 1c). No other cell types in these cultures or in untreated cultures bound tetanus toxin.

The same cells seen in Fig. 1a and c were also exposed to antiserum directed against GFP, a major component of the intermediate filaments found in glial astrocyte cells (16). Figure 1d shows that the monolayer of cells underlying the neurons in Fig. 1a is composed of glial cells. The neurons did not stain with anti-GFP. Thus, neurons and glia form two distinct cell populations in RA-treated cultures. The glial cells did not label uniformly with anti-GFP (Fig. 1d), probably because of the asynchrony with which the glioblasts (GFP^-) mature into astrocytes (GFP^+).

CAT, the enzyme responsible for the synthesis of the neurotransmitter acetylcholine, has been used as a neuronal cell marker (28). This activity was absent from untreated cultures but did appear in RA-treated cultures (Fig. 2b) and peaked at the time when neurons were most numerous. AchE activity showed a coordinate rise (Fig. 2a). The decline in specific activity of these two enzymes at 10 days is probably a consequence of the proliferation of nonneuronal cells in these cultures.

Mechanism of action of RA. The mechanism by which RA induces P19 cells to differentiate into neurons and glial cells may be similar to the mode of action of this drug in other biological systems. Structural analogs of RA have been used to investigate which parts of the RA molecule are important for biological activity (19). A number of retinoids were tested for their ability to induce P19 cells to differentiate into neurons. Table 1 shows that retinoids with major modifications to the C15 carboxylic acid group of RA required much higher concentrations to attain the same efficiency as RA. The relative efficiencies of the analogs are similar to those obtained

TABLE 1. Efficiencies of some retinoids on induction of neuronal development

Retinoid	Half-effective dose ($\times 10^{-8}$ M)[a]	Efficiency relative to all *trans* RA
All *trans* RA	2.8	1
13-*cis* RA	3.8	0.73
Retinal	50	0.06
Retinol	280	0.01
Retinyl acetate	430	0.007
TMMP[b] analog of ethyl retinoate	450	0.006
TMMP analog of *N*-ethyl retinamide	>1,000	

[a] Dose at which 50% of plated aggregates contained neurons.

[b] TMMP, Trimethylmethoxyphenol.

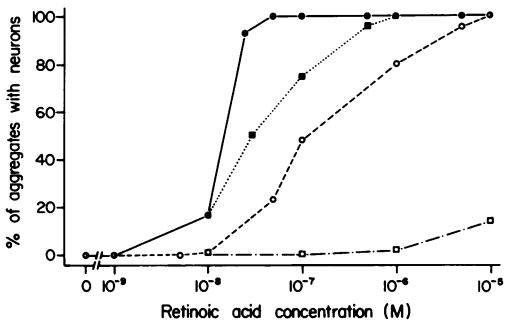

FIG. 3. Failure of RAC65 cells to differentiate into neurons in the presence of RA. Aggregates of cells were cultured for 5 days in the presence of drug, plated, and scored 2 to 3 days later. Normally, 50 aggregates were scored for each drug concentration in each experiment. The points indicate the means obtained from several experiments. Symbols: ●, P19S18; ○, P19S18RAC6; □, RAC65; ■, HY-1.

in some other biological systems (13, 19, 20) and suggest that the intracellular mechanisms of retinoid action in P19 cells are similar to those in those other systems.

RA is an anti-tumor promoter. This activity may result from RA-mediated suppression of ornithine decarboxylase, a key enzyme in polyamine biosynthesis (38). Since polyamines may play a role in some other differentiation systems (8, 32), it seemed possible that the RA effect on P19 could be mediated by decreases in intracellular polyamine levels. Cultures of aggregated P19 cells were exposed to the following drugs, both in the presence and absence of RA: 3×10^{-6} M spermidine, a polyamine; 3×10^{-4} M alpha-methylornithine and 10^{-7} M methylglyoxal-bis-(guanyl-hydrazone), two inhibitors of polyamine biosynthesis; and two tumor promotors, 10^{-5} M phorbol myristate acetate and 3×10^{-6} M dexamethasone. These drug concentrations were nontoxic in 48-h growth tests. None of these drugs had any effect on the P19 cultures, suggesting that changes in polyamine metabolism do not mediate the developmental effects induced in RA.

Mutant cell lines. Since the important biological consequences of RA treatment were not obvious, we used a genetic approach to attempt to determine which events are crucial to the differentiation process. This section describes the stepwise isolation of a cell line which does not differentiate into neurons in the presence of RA. The clone P19S18RAC6 was selected for its ability to grow continuously in the presence of 10^{-7} M RA, and these cells were less responsive to RA than the parental cells (Fig. 3). RAC65 is a subclone of P19S18RAC6 which was isolated in the presence of 10^{-5} M RA. RAC65 cells do not

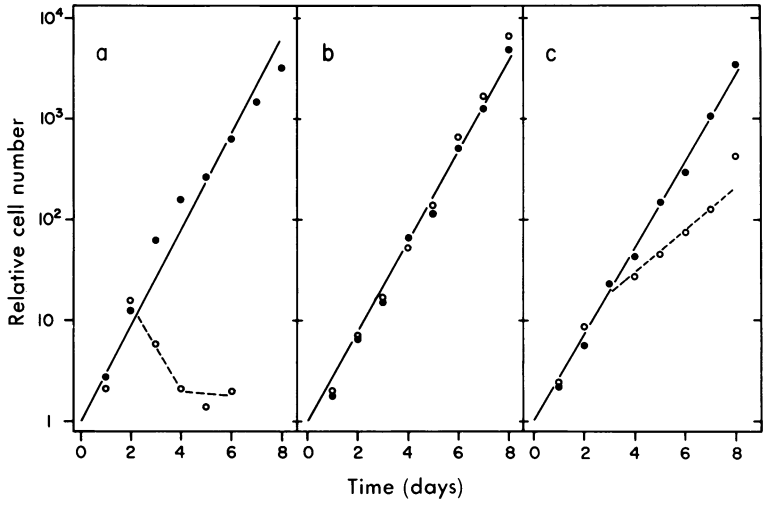

FIG. 4. Continuous growth of RAC65 cells in the presence of 5×10^{-7} M RA. The growth rate of cells in monolayer cultures was determined by plating cells into duplicate tissue culture dishes (10^6 cells per 100-mm dish). The cells from the two dishes were counted after 24 and 48 h. Two new dishes were seeded from the cells in the 48-h dish, and the process was continued for 8 days. The points represent the means of two experiments. The number of cells at each time point was normalized to the cell number at day 0 of the experiment. P19 cells (a), RAC65 cells (b), and HY-1 cells (c) were grown in the continuous presence (○) or absence (●) of RA. The doubling times were as follows: P19, 14.7 h in untreated cultures; RAC65, 16.3 h in treated and untreated cultures; HY-1, 16.7 h in treated and untreated cultures for 3 days, at which time it increased to 20.1 h in treated cultures.

differentiate into neurons at concentrations of RA as high as 10^{-6} M (Fig. 3). RAC65 cells are similar in form to P19 EC cells and have 42 chromosomes.

The RAC65 cells grew continuously and rapidly in both the presence and absence of RA (Fig. 4b), whereas the parental P19 cells did not grow continuously in RA-treated cultures (Fig. 4a). After 2 days in RA, proliferation ceased, and the P19 cells changed into cells with a fibroblastic form. No such morphological change occurred in RA-treated RAC65 cultures.

No cells with the EC form were apparent in RA-treated P19 aggregates by 7 to 8 days after the beginning of the experiment. In contrast, RAC65 aggregates of the same age appeared to consist entirely of undifferentitated cells. To substantiate this observation, we used a mono-

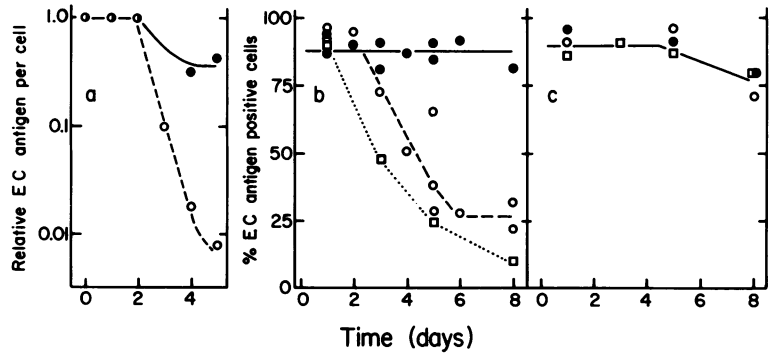

FIG. 5. Disappearance of AEC3A1-9 EC cell-associated antigen from RA- and DMSO-treated aggregate cultures of P19 cells but not from similarly treated RAC65 cells. The relative amount of antigen per P19 cell was determined by an absorption procedure after gluteraldehyde fixation of the cells (a). The percentage of antigen-positive cells in P19 (b) and RAC65 (c) cultures was measured by an indirect immunofluorescence procedure. Each point represents the mean of two or three experiments. Five hundred cells were scored for each point in each experiment of (b) and (c). Symbols: ●, untreated cells; ○, cells treated with 5×10^{-7} M RA; □, cells treated with 1% DMSO.

clonal antibody, AEC3A1-9, which detects an antigen that is found on undifferentiated EC cells but is not present on differentiated cells (Harris et al., submitted for publication). The AEC3A1-9 antigen is closely related to the antigen detected by SSEA-1 (33).

Both RA and DMSO induced the disappearance of antigen-bearing cells from cultures of P19 aggregates (Fig. 5b) but not from cultures of RAC65 aggregates (Fig. 5c). Although about 25% of P19 cells from drug-treated cultured cells carried antigen at 5 days, the fluorescence was weak, and quantitative immunoabsorption experiments indicated that cells in RA-treated cultures contained only about 1% of the antigen concentration present on untreated cells (Fig. 5a). The disappearance of the EC antigen from these cultures was also observed in protein blots (Fig. 6). The antigenic molecules migrated very slowly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and they were essentially absent by day 4.

Cell-cell hybridization experiments were carried out in which RAC65 cells were fused to P19S1801A1, a 6-thioguanine-resistant and ouabain-resistant clone of P19 cells (23). Clones of hybrid cells were selected in HAT medium (17) supplemented with 1.5 mM ouabain. Two clones, HY-1 and HY-2, were examined in detail. Both hybrid lines differentiated into neurons when aggregated in the presence of RA (Fig. 3), although a few undifferentiated cells remained in the aggregates. These cell lines grew more slowly after 3 days in RA (Fig. 4c) but did not abruptly cease proliferation as P19 did (Fig. 4a). The chromosome numbers of both hybrid cell lines varied between 69 and 80; the combined parental number is 82. Thus, the intermediate phenotype of the hybrids may be a consequence of chromosome segregation, leading to the expression of recessive alleles.

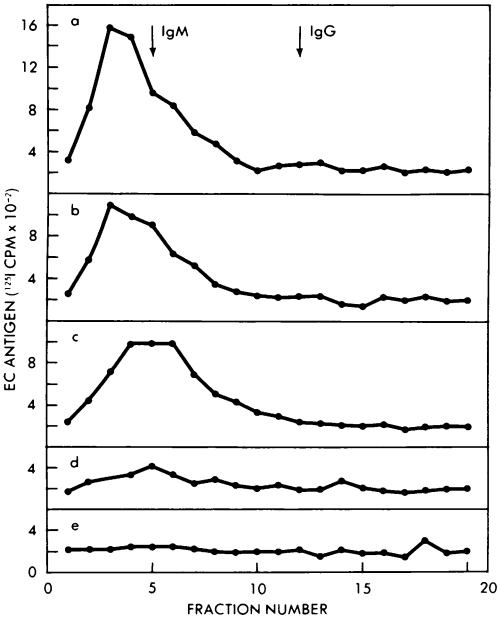

FIG. 6. Decrease in size and amount of AEC3A1-9 antigen in RA-treated cultures. Nonidet P-40 cell extracts were electrophoresed on polyacrylamide slab gels, transferred onto nitrocellulose, and treated with AEC3A1-9 ascites fluid followed by ^{125}I-labeled F(ab')$_2$ rabbit anti-mouse Fab. Pieces (1 by 0.5 cm) of the replica were counted in a Beckman Gamma 4000 counter. Results are shown for antigen from undifferentiated cells (a), 1 day after the addition of 5×10^{-7} M RA to cell aggregates (b), after 2 days in RA (c), after 3 days in RA (d), after 6 days in RA (e). The arrows labeled IgM and IgG are molecular weight markers.

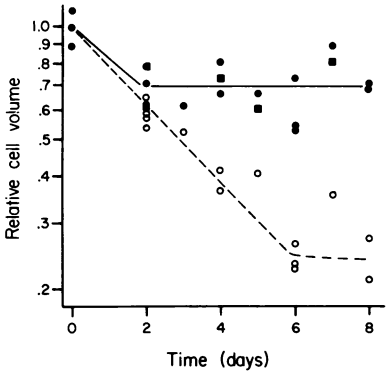

FIG. 7. Decrease in volume of cells from RA-treated aggregates. Aggregated cells were dissociated at daily intervals. Each point represents the median cell volume calculated from a size distribution obtained with a Coulter Counter Channalyser. Symbols: ●, untreated P19 cells; ○, P19 cells treated with 5×10^{-7} M RA; ■, RAC65 cells treated with 5×10^{-7} M RA.

Cell volume changes associated with commitment. The cells in P19 aggregates in RA-treated cultures became smaller than cells in untreated cultures. We estimated the median cell volume from cell size distributions obtained with a Coulter Counter Channalyzer. The 30% decrease in the volume of untreated cells probably reflects accumulation of cells in the G1 phase of the cell cycle. RA-treated cultures showed a much more dramatic 75% decrease in median cell volume (Fig. 7).

This large volume decrease affected all cells in the population and was only seen under conditions in which neurons and glia were destined to develop. RA-treated RAC65 cell aggregates did not show this decrease in cell size (Fig. 7). P19 cells treated with RA in monolayer cultures developed into fibroblasts, but not into neurons and glia. No decrease in median cell volume was observed in these cultures (data not shown).

DISCUSSION

The results reported here confirm the identity of the neural cell types which develop from RA-treated aggregates of P19 cells. The cells which morphologically resemble neurons contain neurofilaments (14) and tetanus toxin binding sites, two neuronal cell markers. The presence of GFP remains the only marker which we have successfully applied to the glial cells, but double-labeling experiments clearly distinguish the astroglia from the neurons. Both neural cell types resemble the embryonic rather than the adult cells because they lack markers of mature neural cells. For example, the neurons do not stain with antibody to Thy-1 glycoprotein (6), and the glial cells do not contain S-100 protein (26) (unpublished observations). The observation that CAT and AchE activities appear coordinately in RA-treated cultures provides further evidence for the neuronal nature of some of the cells formed and may also suggest that some of the neurons are cholinergic. Further studies are under way to explore the character of the neurons more fully.

Our previous work (14) indicated that RA could be removed after 48 h and still be fully effective in inducing neural development. We have shown that shortly after this 48-h period, the cells in treated aggregates begin to lose their EC antigen and their volumes start to decrease. The decrease in cell volume is an early event in neural development of the P19 cultures but is not necessary for all forms of differentiation because no drop in cell volume is seen in DMSO-treated aggregates of P19 cells destined to develop into nonneural tissues, including muscle (3). Decreases in cell volume have been shown to be early events in the commitment of Friend erythroleukemia cells (18) and the spontaneous differentiation of an EC cell line called 1009 (28). The effects noted on RA-treated P19 aggregates are more dramatic because the cells do not simply accumulate in the G1 phase of the cell cycle but shrink to 25% of their original volume. This may suggest that major changes in ion movements accompany the transition of cells from the pluripotent state to the neuroectodermal lineages.

The EC antigen defined by AEC3A1-9 is probably the same molecule recognized by SSEA-1 (33). This antigen exists on molecules which migrate very slowly in sodium dodecyl sulfate-polyacrylimide gels (apparent molecular weight of about 10^6). It disappears more rapidly from DMSO-treated aggregates than from RA-treated aggregates and remains present in small quantities on about 25% of cells in RA-treated cultures. It is possible that the antigen may be synthesized in small amounts by certain differentiated cells or that residual antigen may be trapped after its secretion by the few remaining EC cells.

It remains unclear how RA triggers the neural developmental pathway. RA has a number of diverse biological effects (19). The relative efficiencies of RA analogs are similar in many in vitro systems (19, 20). This hierarchy of efficiencies is correlated with the affinity of the retinoids for the cellular RA-binding protein (13, 20). The effectiveness of the various retinoids that we have tested in the P19 system suggests that the reaction of RA with the cellular RA-binding protein may be a first step in inducing P19 cells to differentiate into neurons. This is consistent with the observations of Schindler et al. (31), who found that a line of RA-nonresponsive PCC4 EC cells lacked cellular RA-binding protein activity. In some systems, RA acts by inhibiting the induction of ornithine decarboxylase, a key enzyme in polyamine biosynthesis (8, 19, 32, 38). However, our data suggest that polyamine biosynthesis is not involved in triggering the neural developmental pathway.

RAC65 cells have maintained their nonresponsive phenotype for many generations in tissue culture, suggesting that one or more stable genetic changes have occurred in these cells. Two selection steps were needed for the isolation of RAC65, and we have been unsuccessful in several attempts to isolate a nonresponsive mutant with only one selection step. Since the inability to respond to RA seems to behave as a recessive character in the cell hybrids, the two-step isolation requirement probably reflects the presence of two active alleles in the diploid P19 cells.

Different concentrations of RA are effective in inducing the development of different cell types (4), and structurally related drugs such as DMSO, butyrate, and 6-thioguanine have similar differentiation-inducing properties to those of RA (3). The fact that RAC65 cells fail to respond to any of these drugs suggests that all these drugs have a common intracellular pathway of action and that the defect(s) in RAC65 cells affects some component of this shared pathway.

ACKNOWLEDGMENTS

Anti-GFP antiserum was a generous gift from V. I. Kalnins. Hoffman Laroche Inc. (Nutley, N.J.) kindly provided the 13-*cis* RA and the TMMP analogs of *N*-ethylretinamide and ethylretinoate. We thank J. Campione-Piccardo for helpful discussions and J. Chin for expert technical assistance.

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada. E.M.V.J.-V. is a research student of the National Cancer Institute of Canada.

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